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Membrane-Assisted Isoelectric Focusing Device As a Micropreparative Fractionator for Two-Dimensional Shotgun Proteomics

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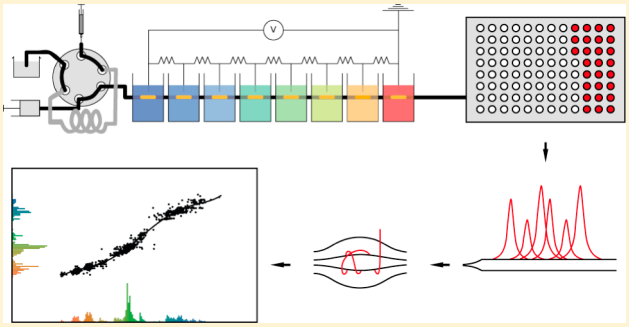
Supporting Information

ABSTRACT: Recently, we introduced an online multijunction capillary isoelectric focusing (OMJ-CIEF) fractionator to fractionate proteins and peptides in electrospray-friendly solution. In this follow-up study, the original configuration of the fractionator was modified to improve the resolving power and reproducibility of separation. The major improvements include stabilization of the electrical current through the device using a voltage divider and stepwise elution of peptide zones in conjunction with the repeated refocusing of remaining peptides. Also, a novel algorithm was developed to calculate more accurately the pI values of peptides identified from experimental data. The standard deviation of calculated pI values for unmodified peptides from the theoretically predicted pI values was on average 0.21 pH units, which is more accurate than in standard-resolution gel-based methods. In order to characterize the analytical performance of the improved device, it was applied for the pI fractionation of yeast proteome digest into 18 fractions, with the collected fractions being analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry. Approximately 37% of 20047 identified peptides were detected in only one fraction and 27% - in two fractions. On average, every peptide was found in 2.4 fractions. These results strongly indicate the suitability of the improved device as a first dimension of separation in multidimensional shotgun proteomics analysis, with a potential for fully automated workflow.



Capillary isoelectric focusing (CIEF) fractionation of proteins and peptides from complex samples has emerged as an attractive technique in many protein studies.[1−7] CIEF separation of polypeptides in solution according to their isoelectric points (pI) can be coupled to online MS detection, generally using an ESI coaxial sheath flow interface.[8,9] For the analyses of particularly complex samples (e.g., protein digests of eukaryotic cells), CIEF is usually coupled to the orthogonal reverse-phase liquid chromatography online with tandem mass spectrometry (RPLC−MS/MS) analysis to cover cell proteome at a reasonable depth.[10,11] Beneficially, the separation in CIEF can be achieved both at the level of protein and peptides. Prefractionation of proteins prior to enzymatic digestion is often employed in deep proteomics studies.[5,12,13]

The final resolution achieved with CIEF is highly dependent not only on the focusing step but also on mobilization conditions. During the past decade, both the optimization and control of these steps have been the subject of many studies.[14,15] Conventional CIEF employs either hydraulic mobilization assisted by pressure, vacuum, or gravity or salt mobilization.[16,17] Originally, a UV-detector installed between the cathode and anode was used for detecting the CIEF fractions. This way, elution of separated analytes from the column was not necessary.[18] Unlike UV detection, MS detection requires the elution of focused zones from the column. Mobilization process induces peak broadening as a new factor that negatively affects the resolving power of CIEF.[19] After the molecules have passed the focusing area, they are not exposed to an electrical field anymore. As a result, diffusion of the analytes inside the capillary gradually deteriorates the achieved pI resolution. The longer the distance between the column and the fraction collector, the more severe is peak broadening. Besides that, analytes passing through the anode or cathode regions can be electro-captured, which further affects the resolution and sensitivity of analysis.[20] In one study, Zhang et al. reported a method to improve mobilization by controlling the flow of eluent and electrical voltage by releasing each peak, while leaving the remaining protein zones focused inside the capillary.[21] This stepwise mobilization has improved the pI resolution, but the improvement is sample dependent, and the precise value of the flow rate

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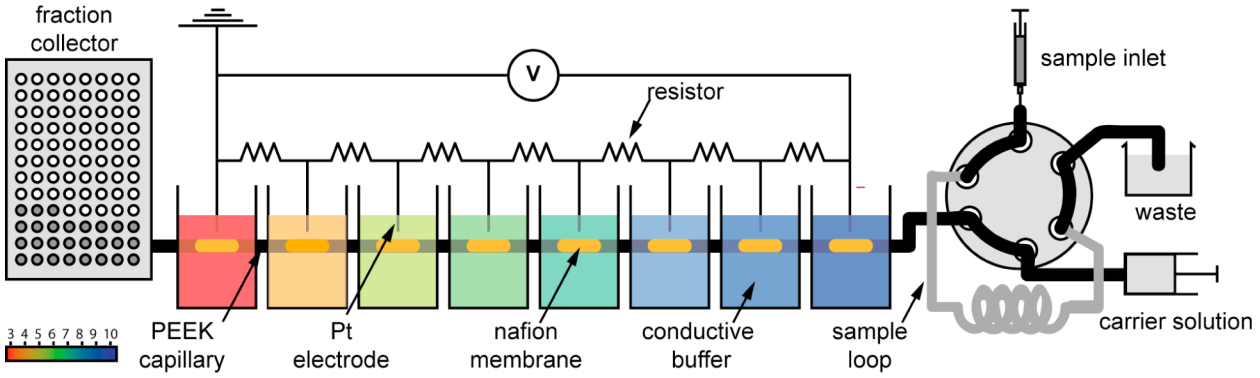

Figure 1. Scheme of the IEF Fractionator Device. The device consists of a 6-port valve, with loading and injection positions, connected to the multijunction IEF column consisting of PEEK capillaries with Nafion membrane windows immersed in electrolytic buffer solutions in vials 1−8. The vials contain solutions with different pH values, thus creating a pH gradient inside the column. Resistors of the voltage divider ensure stable and linear distribution of the electrical field along the column.

and voltage needs to be optimized for each polypeptide mixture. Also, the dynamic flow rate changes the retention time, which adds more complexity to data interpretation.

Running isoelectric focusing at high-loading capacity is challenging in terms of both focusing and mobilization. Focusing is complicated due to the higher Joule heat generation and accumulation of aggregates in in-solution IEF columns with larger diameter.[22] Mobilization is affected by a higher rate of diffusion and turbulent flow.[3] Yet high loading capacity is a prerequisite for the use of IEF in micropreparative devices for shotgun proteomics. Indeed, a single RPLC−MS/MS experiment consumes ca. 0.5−1.0 μg of protein digest. Since in two-dimensional (2D) proteomics, 6−80 fractions are used, the loading capacity of a micropreparative IEF device should be >10 μg.

Recently we have introduced a novel online multijunction capillary isoelectric focusing fractionator (OMJ-CIEF) for the separation of proteins and peptides in solution.[23] The 10 cm separation capillary in OMJ-CIEF is made of PEEK with an o.d. of 635 μm and an i.d. of 395 μm and has an internal volume of 12 μL. Focusing performance of OMJ-CIEF in ESI-friendly solutions has been proven for the model mixtures of proteins and peptides. Multicompartment assembly of the tool provides the ability of stabilizing pH gradient via permeable membranes on each junction, which resulted in low sample contamination by carrier ampholytes and well-focused zones.

In the current study, we optimized the OMJ-CIEF as a micropreparative device for fractionation by pI of >10 μg of complex peptide mixtures. For the better linearity and stability of the electrical field across the fractionator, a voltage divider was implemented with an electrode in every cell, as detailed in Experimental Section (Figure 1). To reduce the peak broadening during the elution of the focused zones, a new stepwise focusing and mobilization strategy is implemented, with refocusing of the remaining in the device volume after each elution step. The optimized method offers increased resolution, which was demonstrated in the fractionation of a peptide mixture from the model system, yeast proteome. To fully utilize the analytical performance of the improved device, a novel algorithm was developed for pI calculation of identified peptides and post-translational modifications based on the elution time information. Thus, additional, analytically valuable information on the pI values of identified peptides and potentially valuable information on post-translational modifications (PTMs) was obtained.

EXPERIMENTAL SECTION

Details on assembling of the CIEF fractionator, voltage divider, fractionation protocol, RPLC−MS/MS experiment, pI calculation, and experimental pI determination as well as chemicals and materials used in this study can be found in the Supporting Information. Briefly, a solution containing 20 g/L tryptic peptide mixture from digested yeast proteome was loaded into a 2 μL loop and separated within 25 min into 18 fractions. The fractions were loaded onto a column of a nanoflow uPLC instrument connected online with a Q Exactive mass spectrometer. A conventional liquid chromatography−tandem mass spectrometry (LC−MS/MS) experiment was performed, with peptide identification and quantification performed with MaxQuant.[24]

RESULTS AND DISCUSSION

Effect of Pharmalyte Carry-Over from IEF to LC−MS/MS. Carrier ampholytes (CA) are a family of chemical mixtures, commercially manufactured for creating and maintaining the pH gradient in the presence of an electric field.[25] CAs are amphoteric soluble mixtures consisting of oligoamino and oligocarboxylic acids, usually available for both narrow and wide ranges of pH gradients.[3] Pharmalyte is one of the carrier ampholytes widely used for isoelectric focusing experiments.

However, using ampholytes (particularly, Pharmalyte) in IEF risked introducing negative effects into LC−MS/MS analysis due to an increased background of unrelated ions. Therefore, the Pharmalyte effect on the LC−MS/MS proteomics experiment was studied by comparing the number of identified peptides from the yeast proteome digest spiked with different concentrations of Pharmalyte, from 0% (control) to 5% in a direct LC−MS/MS experiment without prior IEF separation. Figure 2 shows that the identification rate is significantly affected (negatively) by Pharmalyte concentration of 0.5% and higher, while at lower concentration the results are similar to control.

Given the positive effect of ampholytes on IEF, in the rest of this study we used 0.5% Pharmalyte concentration for peptide fractionation. The eluted fractions were diluted six times in buffer A before injection into RPLC, and thus the final Pharmalyte concentration in the LC−MS/MS run was below 0.1%. At such a low concentration of Pharmalyte, the separated fractions did not require additional cleanup and could be directly injected in RPLC for 2D shotgun proteomics analysis.

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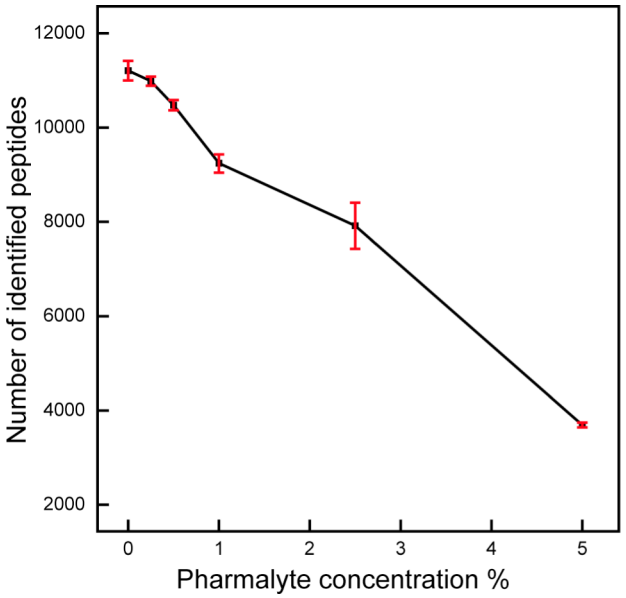

Figure 2. Pharmalyte effect on RPLC–ESI-MS/MS results. Two micrograms of yeast digest dissolved in 5 μL were spiked with 0%, 0.25%, 0.5%, 1.0%, 2.5% and 5% (v/v) Pharmalyte, pH 3–10. Each sample was analyzed with RPLC coupled with MS/MS. The acquired data was analyzed and an identified number of tryptic peptides plotted for each concentration. Error bars represent the standard deviation for three replicate analyses.

Electrical Current Stabilization. The driving force in IEF is the electrical field, which forces the charged compounds to move along the pH gradient until they reach the pH region where they are no longer charged and thus become focused there. It is generally agreed that a linear and strong electrical field is the precondition of a successful IEF experiment.[26] The field strength and profile depend upon the applied voltage and the conductivity of the media, including the running buffer, analyte, other ions and salts present, as well as the proton and hydroxyl ions that eventually accumulate on the anode and cathode during the CIEF experiment. These latter effects can affect the local conductivity of the media and consequently the electrical field. Moreover, since the focusing speed of different analytes in a mixture varies, as soon as an analyte component is focused in the column, it forms a strong nonconductive barrier, forcing the field to concentrate in conductive regions of the column. This phenomenon known as hot spots in CIEF,[27] negatively affects the analytical performance of the CIEF devices, as it reduces the effective length of the analytical column.

To avoid these problems and establish a stable linear electrical field during the experiment, we made a resistor-based voltage divider (Figure 1). The divider ensures the same voltage drop across each section. This improves the separation performance, avoids generation of hot spots, as well as preserves the effective length of the column.

Figure 3 (panels a and b) compare two 2D proteomics experiments performed with and without the voltage divider. The experimental pI scale was calibrated using a polynomial. After optimizing the polynomial order (Figure S2 of the Supporting Information), it was found that fifth order gives optimal performance, providing a trade-off between simplicity and accuracy.

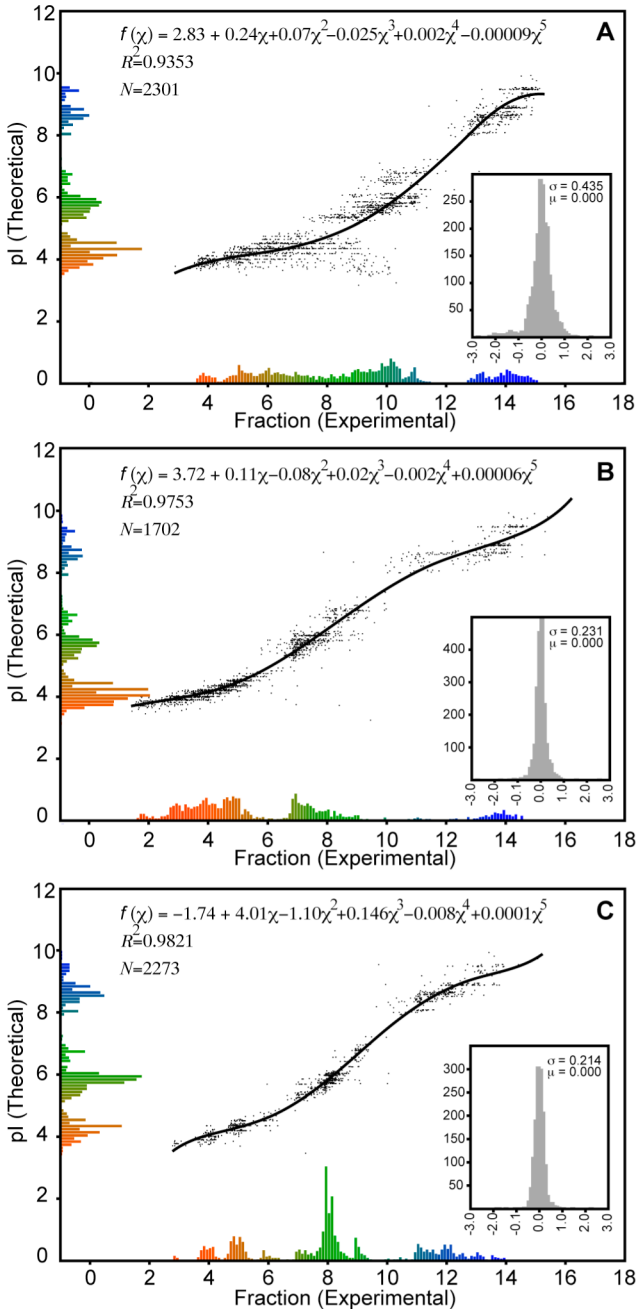

Figure 3. Experimentally determined pI versus theoretical pI and the corresponding error distributions. (A) Neither voltage divider nor refocusing was applied: σ = 0.435 (15044 peptides identified in total). (B) Voltage divider was used, but no refocusing: σ = 0.231 (17991 peptides). (C) Both voltage divider and refocusing were used, σ = 0.214 (20047 peptides). Peptide elution "time" is determined in fractions, and theoretical values are calculated as described Experimental Section. A polynomial calibration curve of power 5 was fitted using data for unmodified peptides without cysteine. σ value is the standard deviation of the experimental pI from the fitted curve.

With the divider, the separation performance is improved, and the experimentally determined pI values are on average closer to their theoretical pI values, as witnessed by the σ value that decreased from 0.44 to 0.23.

Refocusing. When focused zones are released from the column (mobilized), the first eluted zones are sharp, but later, the eluting zones are getting increasingly broader while traveling

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along the column due to diffusion and turbulent flow.[23] To reduce the peak broadening of focused zones during their mobilization, we applied a stepwise refocusing procedure. After the focusing step, the first anode zone (acidic peptides) is eluted from the column, while the rest of the zones are subjected to refocusing within the remaining part of the column. Such refocusing not only sharpens the broadened peaks but also improves the focusing of late-eluting basic polypeptides, which is a challenge in IEF performed at a wide pH range. The results shown in Figure 3 (panels b and c) illustrate the resolution improvement due to refocusing by comparing the corresponding σ values in an experiment with and without refocusing: the σ value decreased from 0.23 to 0.21.

The improvement from refocusing was not only in the reduced global variation but also in a significantly reduced spread of the elution time of basic (late-eluting) peptides. Thus, with refocusing, basic peptides were more enriched in the respective fractions, which provided a better chance for their identification. Consistent with this, more peptides (20047) were identified in the 2D proteomics experiment with refocusing than without it (17991). Without refocusing, the pI distribution of detected peptides was skewed toward acidic peptides, as the lower abundance of the basic peptides made their detection less probable.

pI Predictions and PTMs. Peptide's pI value provides additional, and often valuable, information for peptide identification.[28−30] Consequently, several algorithms have been developed for pI-prediction of peptides based on their sequence,[31−35] amino acid substitutions, or modifications.[36] There are about 200 types of post-translational modifications identified in the human proteome.[37] Characterization of post-translational modifications is one of the challenging tasks in proteomics studies, not least because most PTMs are present in substoichiometric quantities. In a typical proteomics experiment, the acquired MS/MS spectra of peptides are searched against a protein sequence database and a match score indicates the quality of sequence assignment. Considering PTMs as possible (variable) modification manifolds increases the time needed for the search and significantly elevates the risk of false positive sequence identification. To deal with the problem, more sequence-specific information needs to be added to the MS/MS data set. For that, several methods have been introduced, such as MS/MS/MS (MS^3) sequencing[38] or the use of high accuracy of the measured precursor ion mass to limit the search space in the database search.[39] Additionally, experimentally determined peptide pI value has been used for more reliable peptide and PTM identification.[40,41]

In this study, we investigated the pI shift due to two important and well-studied PTMs, deamidation of Asn and phosphorylation on Ser, Thr, or Tyr. To calculate the pI shift for each peptide, the exact focusing position of unmodified and modified species of the peptide were calculated. By means of fitting a curve in Figure 3c, pI of each peptide species was estimated. The difference between the pI values of modified and unmodified peptide represents the pI shift due to modification. As Figure 4 illustrates, the pI shift of the peptides due to both of these modifications is far from constant and depends upon the pI of the unmodified peptide.

Therefore, experimentally determined pI values and ΔpI shifts due to PTM can provide additional sequence-specific information and thus help reducing the false discovery rate in shotgun proteomics. This information can be applied for pI-based FDR filtration, as an extra parameter for rejecting false hits in the database search.

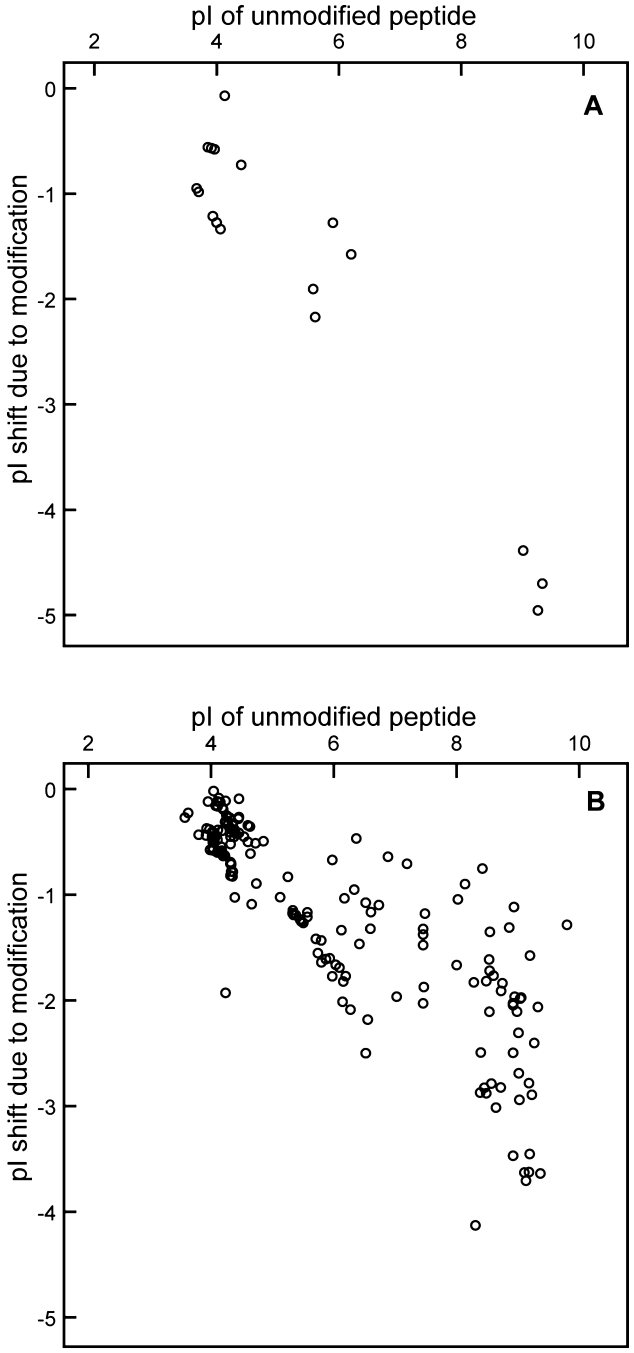

Figure 4. pI shift due to a single post-translational modification. (A) phosphorylation of Ser, Thr, or Tyr. (B) Asn deamidation.

Fractional Resolution. What is the minimal number of pI fractions in a 2D proteomics experiment that preserves the analytical power of the pI-based separation? To address this question, the distribution of peptide occurrences in multiple fractions of the data set in Figure 3c was investigated. It peaks at one occurrence (Figure S3 of the Supporting Information), with ca. 37% of 20047 peptides identified in this 2D IEF-LC−MS/MS experiment being detected in only one fraction and 27% in two fractions. An average peptide was found in 2.4 fractions. Since the maximum number of peptides detected in a single fraction was 4500, ∼11 fractions [obtained as (20047 × 2.4)/

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4500] would be sufficient to provide maximum separation of the peptide mixture in Figure 3C, if the peptide distribution across different fractions was homogeneous. The pI distribution of tryptic peptides is however strongly inhomogeneous, with several peaks separated by deep valleys (see distributions on the *y* scale in Figure 3). A similar distribution was also found in previous studies.[42] Adding 30% for this inhomogeneity, arrives at 14−16 fractions per proteome as a practical optimum.

CONCLUSION

High-capacity isoelectric focusing is a nearly ideal first dimension of fractionation before the conventional RPLC in shotgun proteomics. The improvements made in the earlier developed OMJ-CIEF fractionator make it a suitable device for preparative fractionation of real shotgun proteomics samples, such as yeast or mammal cell digests. The separation is fast, user-friendly, low cost, automatable, and with minimal contamination of the sample and instrument by salts, buffers, and ampholytes. Future integration of this device into available autosampler for RPLC will provide a fully automatic method for 2D shotgun proteomics.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Fonslow, B. R.; Kang, S. A.; Gestaut, D. R.; Graczyk, B.; Davis, T. N.; Sabatini, D. M.; Yates, J. R. *Anal. Chem.* **2010**, *82*, 6643−6651.

(2) Chen, J. Z.; Balgley, B. M.; DeVoe, D. L.; Lee, C. S. *Anal. Chem.* **2003**, *75*, 3145−3152.

(3) Righetti, P. G.; Sebastiano, R.; Citterio, A. *Proteomics* **2013**, *13*, 325−340.

(4) Wang, W. J.; Guo, T.; Song, T.; Lee, C. S.; Balgley, B. M. *Proteomics* **2007**, *7*, 1178−1187.

(5) Zhou, F.; Johnston, M. V. *Electrophoresis* **2005**, *26*, 1383−1388.

(6) Haselberg, R.; de Jong, G. J.; Somsen, G. W. *J. Chromatogr. A* **2007**, *1159*, 81−109.

(7) Zhu, G. J.; Sun, L. L.; Yang, P.; Dovichi, N. J. *Anal. Chim. Acta* **2012**, *750*, 207−211.

(8) Chartogne, A.; Tjaden, U. R.; Van der Greef, J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1269−1274.

(9) Martinovic, S.; Berger, S. J.; Pasa-Tolic, L.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 5356−5360.

(10) Washburn, M. P.; Wolters, D.; Yates, J. R. *Nat. Biotechnol.* **2001**, *19*, 242−247.

(11) Beck, M.; Schmidt, A.; Malmstroem, J.; Claassen, M.; Ori, A.; Szymborska, A.; Herzog, F.; Rinner, O.; Ellenberg, J.; Aebersold, R. *Mol. Syst. Biol.* **2011**, *7*.

(12) Malmstrom, J.; Lee, H.; Nesvizhskii, A. I.; Shteynberg, D.; Mohanty, S.; Brunner, E.; Ye, M. L.; Weber, G.; Eckerskorn, C.; Aebersold, R. *J. Proteome Res.* **2006**, *5*, 2241−2249.

(13) Busnel, J. M.; Lion, N.; Girault, H. H. *Anal. Chem.* **2007**, *79*, 5949−5955.

(14) Renato, M.; Cinzia, F.; Paolo, T.; Rita, P.; Daniela, C.; Giorgio, A. *J. Chromatogr. A* **2013**, *1293*, 1−9.

(15) Kasicka, V. *Electrophoresis* **2012**, *33*, 48−73.

(16) Cao, C. X.; Fan, L. Y.; Zhang, W. *Analyst* **2008**, *133*, 1139−1157.

(17) Wehr, T. In *Handbook of Isoelectric Focusing and Proteomics*; Elsevier: Amsterdam, 2005; pp 181−210.

(18) Thomassen, Y. E.; van Eikenhorst, G.; van der Pol, L. A.; Bakker, W. A. M. *Anal. Chem.* **2013**, *85*, 6089−6094.

(19) Kang, D. J.; Moon, M. H. *Anal. Chem.* **2006**, *78*, 5789−5798.

(20) Astorga-Wells, J.; Tryggvason, S.; Vollmer, S.; Alvelius, G.; Palmberg, C.; Jornvall, H. *Anal. Biochem.* **2008**, *381*, 33−42.

(21) Zhang, C. X.; Xiang, F.; Pasa-Tolic, L.; Anderson, G. A.; Veenstra, T. D.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 1462−1468.

(22) Kates, B.; Ren, C. L. *Electrophoresis* **2006**, *27*, 1967−1976.

(23) Chingin, K.; Astorga-Wells, J.; Najafabadi, M. P.; Lavold, T.; Zubarev, R. A. *Anal. Chem.* **2012**, *84*, 6856−6862.

(24) Cox, J.; Mann, M. *Nat. Biotechnol.* **2008**, *26*, 1367−1372.

(25) Laas, T.; Olsson, I.; Soderberg, L. *Anal. Biochem.* **1980**, *101*, 449−461.

(26) Huang, T. M.; Wu, X. Z.; Pawliszyn, J. *Anal. Chem.* **2000**, *72*, 4758−4761.

(27) Takacsi-Nagy, A.; Kilar, F.; Pager, C.; Mosher, R. A.; Thormann, W. *Electrophoresis* **2012**, *33*, 970−980.

(28) Cargile, B. J.; Sevinsky, J. R.; Essader, A. S.; Stephenson, J. L., Jr.; Bundy, J. L. *Journal of Biomolecular Techniques: JBT* **2005**, *16*, 181−189.

(29) Krijgsveld, J.; Gauci, S.; Dormeyer, W.; Heck, A. J. R. *J. Proteome Res.* **2006**, *5*, 1721−1730.

(30) Heller, M.; Ye, M. L.; Michel, P. E.; Morier, P.; Stalder, D.; Junger, M. A.; Aebersold, R.; Reymond, F. R.; Rossier, J. S. *J. Proteome Res.* **2005**, *4*, 2273−2282.

(31) Bjellqvist, B.; Hughes, G. J.; Pasquali, C.; Paquet, N.; Ravier, F.; Sanchez, J. C.; Frutiger, S.; Hochstrasser, D. *Electrophoresis* **1993**, *14*, 1023−1031.

(32) Shimura, K.; Zhi, W.; Matsumoto, H.; Kasai, K. *Anal. Chem.* **2000**, *72*, 4747−4757.

(33) Cargile, B. J.; Sevinsky, J. R.; Essader, A. S.; Eu, J. P.; Stephenson, J. L. *Electrophoresis* **2008**, *29*, 2768−2778.

(34) Gauci, S.; Van Breukelen, B.; Lemeer, S. M.; Krijgsveld, J.; Heck, A. J. R. *Proteomics* **2008**, *8*, 4898−4906.

(35) Perez-Riverol, Y.; Audain, E.; Millan, A.; Ramos, Y.; Sanchez, A.; Vizcaino, J. A.; Wang, R.; Muller, M.; Machado, Y. J.; Betancourt, L. H.; Gonzalez, L. J.; Padron, G.; Besada, V. *J. Proteomics* **2012**, *75*, 2269−2274.

(36) Lengqvist, J.; Eriksson, H.; Gry, M.; Uhlen, K.; Bjorklund, C.; Bjellqvist, B.; Jakobsson, P. J.; Lehtio, J. *Amino Acids* **2011**, *40*, 697−711.

(37) Savitski, M. M.; Nielsen, M. L.; Zubarev, R. A. *Mol. Cell. Proteomics* **2006**, *5*, 935−948.

(38) Olsen, J. V.; Mann, M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13417−13422.

(39) Zubarev, R.; Mann, M. *Mol. Cell. Proteomics* **2007**, *6*, 377−381.

(40) Essader, A. S.; Cargile, B. J.; Bundy, J. L.; Stephenson, J. L., Jr. *Proteomics* **2005**, *5*, 24−34.

(41) Krijgsveld, J.; Gauci, S.; Dormeyer, W.; Heck, A. J. *J. Proteome Res.* **2006**, *5*, 1721−1730.

(42) Weiller, G. F.; Caraux, G.; Sylvester, N. *Proteomics* **2004**, *4*, 943−949.